褐藻マツモより一新ペプチド,アナリピンの単離および2,3の性質について

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Isolation and Some Chemical Properties of a New Peptide, Analipine, from a Brown Alga, *Analipus japonicus**

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A new peptide was found in the 75% ethanol extracts of a brown alga, *Analipus japonicus*. The peptide was isolated as cross-shaped crystals by gel filtration through Sephadex G–10, ion exchange chromatography on Dowex 50 resin, and mercuric acetate precipitation. The peptide appears to be a pentapeptide consisting of glutamic acid and aspartic acid or their amides, since it gave 1 mole of glutamic acid and 4 moles of aspartic acid on acid hydrolysis. The peptide was named "analipine" after the generic name of the alga.

Although many studies on peptides of marine algae have hitherto been performed by a number of researchers[1–9], only a few peptides have been isolated purely, namely, eisenine from *Eisenia bicyclis*[10,11], fastigiatine from *Pelvetia fastigiata*[5], and L-arginyl-L-glutamine from a freshwater species of *Cladophora*[12].

KONAGAYA[13,14] has recently isolated eisenine from *Ecklonia cava*, a brown alga closely related to *Eisenia bicyclis*. However, he suggested that the pyrrolidone ring of eisenine must have been secondarily formed during the course of its isolation, as DNP-derivative of Gln-Gln-Ala could be obtained from an aqueous extract of *Ecklonia cava*.

The present paper deals with the isolation and some chemical properties of a new peptide, "analipine", from a brown alga, *Analipus japonicus*.

Experimental and Results

Material The sample of *A. japonicus* was collected on the rocky reef at Shinori in Hakodate on March 10, 1972, and stored at −20°C.

Isolation of analipine** Four kg of the frozen material was soaked and stirred occasionally in 5 liters of 75% ethanol for 3 days at room temperature. After filtration, the residue was extracted once more in the same manner. The combined extracts were evap-

* The content of this article was first delivered at the meeting of the Japanese Society of Scientific Fisheries, held in Tokyo in April 1971 and again at the meeting of the same Society, held in Kochi in October 1972 on the occasion of crystallization of "analipine". At the former meeting it was named tentatively "abietinine" after the previous specific name of the alga. However, we adopted analipine in the present paper, as the scientific name was revised to *Analipus japonicus* by WYNNE[15] in 1971.

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**** In the course of isolation, concentration of solution was carried out under reduced pressure at 55°C.

orated to about 2 liters, decolourized with 100 g of charcoal treated with 5% acetic acid, and filtered through paper pulp. The resultant light yellow solution was added onto a column of Dowex 50 X-4 (H^+, 800 m*l*, 100–200 mesh), and the column was rinsed with 250 m*l* of distilled water to remove mineral salts and other impurities. The adsorbed substances were eluted with 0.2 N NH_4OH solution and the eluate was concentrated to 200 m*l*.

An aliquot (10 m*l*) of the concentrated solution was applied to a column of Sephadex G-10 (5×100 cm, 1000 m*l*) and water was allowed to flow through the column at a rate of 30 m*l* per hr, the eluate being collected in 3 m*l* cuts. To 0.1 m*l* of each fraction 2 m*l* of 2% ninhydrin dissolved in the mixture of equal amount of methylcellosolve and sodium citrate buffer[16] of pH 5.0 and 0.1 m*l* of stannous chloride solution[16] were added. After the

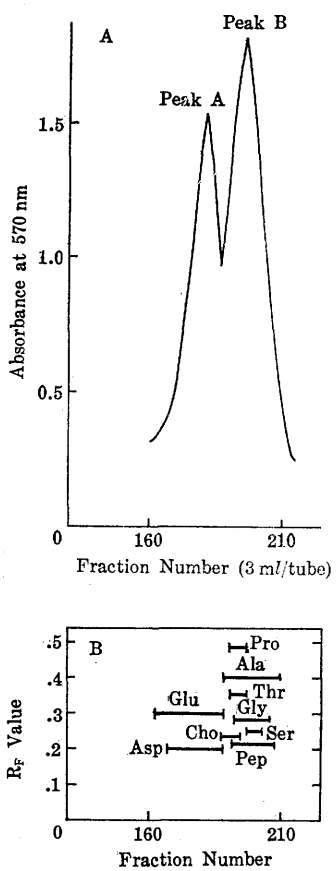

Fig. 1. Gel filtration on Sephadex G-10 of the extract of *Analipus japonicus*. The column (5×100 cm) was equilibrated with water and elution was carried out at 30 m*l* per hr. A, absorbance at 570 nm after ninhydrin reaction. B, paper chromatography.

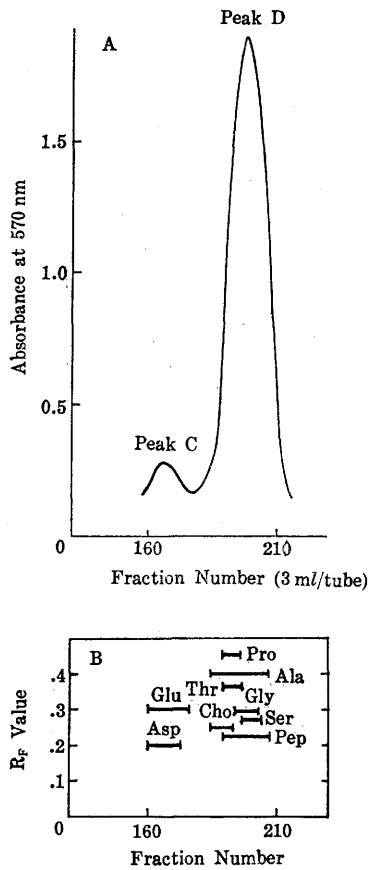

Fig. 2. Gel filtration on Sephadex G-10 of peak B in Fig. 1. The condition was the same as that described in Fig. 1. A, absorbance at 570 nm after ninhydrin reaction. B, paper chromatography.

mixture was heated for 20 min in a boiling water bath and diluted with 10 m*l* of *n*-butanol–water (1 : 1), ninhydrin colour value was measured at 570 nm. Each fraction was also examined by paper chromatography with *n*-butanol–acetic acid-water (4 : 2 : 1, V/V) and Toyo No. 51 paper. Most of aspartic and glutamic acids were eliminated by this treatment (Fig. 1). The gel filtration was carried out 20 times in total, and about 1.5 liters of the pooled solution of peak B was obtained. The above solution was then concentrated to 100 m*l* and subjected to the gel filtration on Sephadex G-10 again in the same condition. As shown in Fig. 2, peak D which contains analipine is completely free from aspartic and glutamic acids. The gel filtration was carried out 10 times in total, and about 900 m*l* of the solution of peak D was collected.

After the solution of peak D was concentrated to 60 m*l*, an aliquot (5 m*l*) was applied

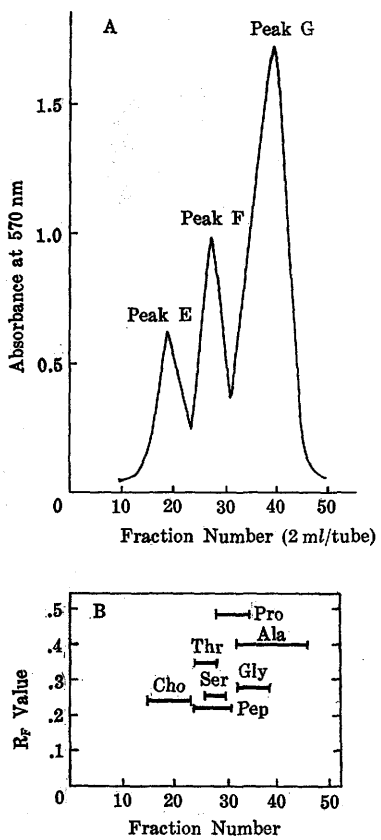

Fig. 3. Ion exchange chromatography of peak D in Fig. 2. Resin, Dowex 50 X-4 (Na^+); column, 0.9×50 cm; buffer, 0.2 N Na-citrate (pH 3.25); temperature, 37°C. A, absorbance at 570 nm after ninhydrin reaction. B, paper chromatography.

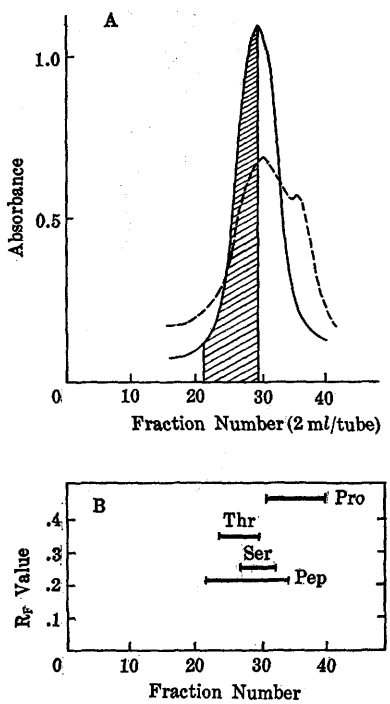

Fig. 4. Ion exchange chromatography of peak F in Fig. 3. The condition was the same as that given in Fig. 3 except that 0.2 N Na-citrate buffer of pH 2.8 was used. A, absorbance at 570 nm (—) and 420 nm (---) after ninhydrin reaction. B, paper chromatography.

to a column of Dowex 50 X-4 (Na^+, 0.9×50 cm, 100–200 mesh) equilibrated previously with 0.2 N sodium citrate buffer of pH 3.25. Elution was carried out with the same buffer at 37°C at a flow rate of 24 m*l* per hr and 2 m*l* fractions were collected. A typical elution pattern is shown in Fig. 3. Analipine is eluted in peak F together with threonine, serine, and a part of proline. By repeating this ion exchange chromatography 12 times in total, about 170 m*l* of the peptide fraction was collected. The fraction was then concentrated to 30 m*l* and subjected to the ion exchange chromatography in the same condition as above to remove proline. The result of a typical experiment is shown in Fig. 4. Although the peptide and proline are overlapping partially, Frs. No. 22 to 29, the shaded portion in the figure, are destitute of proline. So these fractions were pooled. The ion exchange chromatography was carried out 5 times more. From Frs. No. 30 to 34, the peptide devoid of proline was recovered by the same column chromatographic method. In this way 100 m*l* of the peptide solution completely free from proline could be obtained.

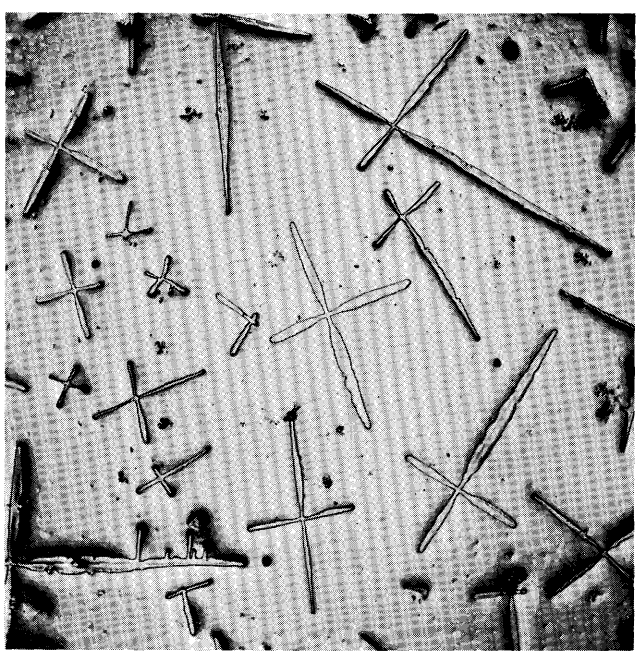

Fig. 5. Crystals of analipine (×100).

The peptide solution was passed through a column of Dowex 50 X-4 (H^+, 0.9×50 cm), and the column was washed with 50 m*l* of distilled water to remove sodium citrate. The peptide was then eluted with 0.2 N NH_4OH solution. The eluate was adjusted to pH 9 with N NH_4OH, and added with 10 m*l* of 5% mercuric acetate. The precipitate formed was filtered, suspended in 20 m*l* of water, and decomposed with H_2S. The precipitate of mercuric sulfide was filtered off.

Table 1. Paper chromatography of analipine.

Solvents	R_F values	Colour
n-Butanol-acetic acid-water (4: 2: 1)	0.22	blue purple
n-Butanol-acetic acid-water (4: 1: 5) upper phase	0.15	purple brown
n-Butanol-ethanol-water (4: 1: 2)	0.13	blue purple
Pyridine-water (4: 1)	0.14	purple brown
Pyridine-*n*-butanol-water (2: 1: 1)	0.13	purple brown
Collidine-lutidine (1: 3)	0.02	purple
Lutidine-aniline-water (9: 1: 2)	0.10	purple
Phenol, water saturated	0.08	purple
Benzyl alcohol, water saturated	0.01	purple

Paper, Toyo No. 51.

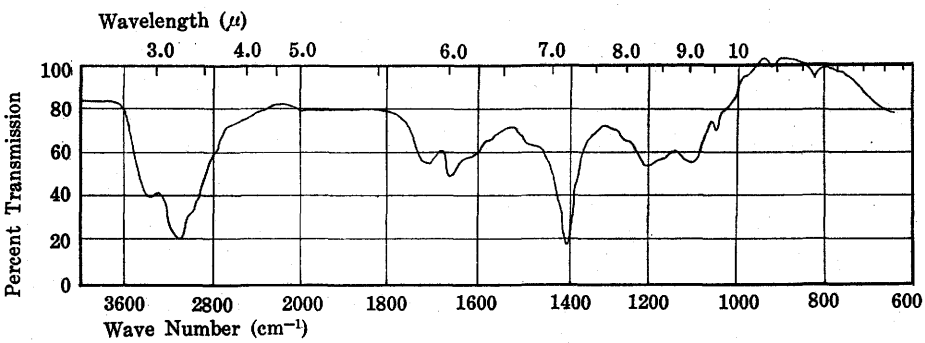

Fig. 6. Infrared spectrum of analipine.

By these treatments threonine and serine could be removed completely and the peptide solution free from any ninhydrin-positive impurities was obtained. The solution was evaporated to dryness in a vacuum desiccator and analipine was crystallized from 85% ethanol, of which yield was about 50 mg. Fig. 5 is a photomicrograph showing the cross-shaped crystals of analipine.

Properties of analipine Analipine had m. p. 197–199°C. Paper chromatography of analipine was performed by the descending technique on Toyo No. 51 paper. A 0.1% solution of ninhydrin in *n*-butanol was used for colour development (Table 1). The preparation of analipine gave only one

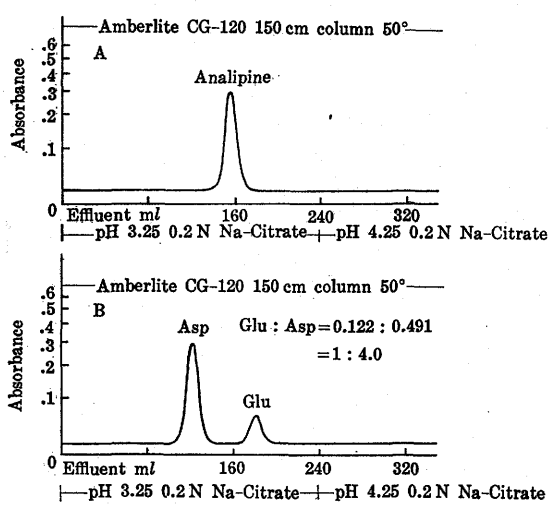

Fig. 7. Amino acid composition of analipine. A, before hydrolysis. B, after hydrolysis.

spot in various solvent systems used.

Infrared spectrum of analipine is given in Fig. 6. It shows a strong absorption band at 1404 cm^{-1} and a weak one at 1665 cm^{-1}.

Analipine (0.2 mg) was hydrolysed in a sealed tube with 6 N HCl (2.5 m*l*) at 105°C for 24 hr, and HCl was removed by repeated evaporations to dryness *in vacuo*. The residue was dissolved in water (10 m*l*) and an aliquot (4 m*l*) was subjected to the Hitachi amino acid analyzer. As shown in Fig. 7, 0.08 mg of analipine gave 0.018 mg of glutamic acid (0.12 μ mole) and 0.065 mg of aspartic acid (0.49 μ mole). Consequently, it seems that analipine is a pentapeptide consisting of glutamic acid and aspartic acid or their amide.

Discussion

Analipine was extracted from the alga with 75% ethanol instead of hot water which has been used widely for extraction of algal peptides[1-5]. The use of the aqueous ethanol has advantages that 1) the extraction of the peptide is feasible at room temperature, 2) the concentration of extracts is easy at lower temperature, and 3) the deproteinizing process is excluded. Thus the formation of pyrrolidone ring in N-terminal glutaminyl residue[14], if present, might be avoidable during the extraction and subsequent concentration processes.

The techniques of gel filtration and column chromatography with bufferized resin were introduced for the first time for isolation of the algal peptide. Although most of impurities could be eliminated easily by these methods, further improvements should be necessary for the preparation of a larger amount of the peptide. At the final stage of the purification, analipine was precipitated with mercuric acetate at pH 9 to remove contaminated threonine and serine. DEKKER *et al.*[5] precipitated fastigiatine with mercuric acetate from acidified solution. In our experiments, however, the precipitation of the peptide was incomplete in acidic media.

The biuret reaction of analipine was found to be negative, probably due to the limited amount of the sample used. However, the amino acid analysis of its hydrolysate clearly showed that it is a peptide consisting of glutamic and aspartic acids or their amides. Further chemical properties and the detailed structure of analipine must await the succeeding study.

Acknowledgements

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